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REVIEW ARTICLE

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Novel strategies to assess cytokine release mediated by chimeric antigen receptor T cells based on the adverse outcome pathway concept

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ABSTRACT

The success of cellular immunotherapies such as chimeric antigen receptor (CAR) T cell therapy has led to their implementation as a revolutionary treatment option for cancer patients. However, the safe translation of such novel immunotherapies, from non-clinical assessment to first-in-human studies is still hampered by the lack of suitable *in vitro* and *in vivo* models recapitulating the complexity of the human immune system. Additionally, using cells derived from human healthy volunteers in such test systems may not adequately reflect the altered state of the patient's immune system thus potentially underestimating the risk of life-threatening conditions, such as cytokine release syndrome (CRS) following CAR T cell therapy. The IMI2/EU project imSAVAR (immune safety avatar: non-clinical mimicking of the immune system effects of immunomodulatory therapies) aims at creating a platform for novel tools and models for enhanced non-clinical prediction of possible adverse events associated with immunomodulatory therapies. This platform shall in the future guide early non-clinical safety assessment of novel immune therapeutics thereby also reducing the costs of their development. Therefore, we review current opportunities and challenges associated with non-clinical *in vitro* and *in vivo* models for the safety assessment of CAR T cell therapy ranging from organ-on-chip models up to advanced biomarker screening.

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Introduction

The approval of engineered CD19-specific chimeric antigen receptor (CAR) T cells for the treatment of relapsed/refractory B cell malignancies has revolutionized the field of cancer immunotherapy (Kalos et al. 2011; Grupp et al. 2013; Maude et al. 2014; Bhoj et al. 2016; Schuster et al. 2017; Maude et al. 2018; Schuster et al. 2019; Vucinic et al. 2021; Melenhorst et al. 2022). CAR T cells combine the specificity of an antigen-binding domain of monoclonal antibodies (mAb) with the cytotoxic and memory function of T cells. The intracellular domain allows the activation of T cells and incorporates stimulatory and co-stimulatory domains such as CD28 or 4-1BB (Locke et al. 2020). The binding process is independent of the major histocompatibility complex (MHC) so that target cell recognition is unaffected by some of the major mechanisms by which tumors avoid MHC-restricted T cell recognition, such as down-regulation of human leukocyte antigen (HLA) Class I molecules and defective antigen processing (Davila et al. 2012; Caruana et al. 2014) (illustrated in Figure 1). Second-generation CAR T cells, like the commercially available axicabtagene ciloleucel (Yescarta®) and tisagenlecleucel (Kymriah®), are equipped with the CD3ζ chain in combination with a CD28 or 4-1BB co-stimulatory domain that allow for prolonged persistence *in vivo* and improved cytokine production (Freyer 2018; Maude et al. 2018; Locke et al. 2019; Ali et al. 2020).

Despite the impressive response rates and long-lasting remissions, short-term serious side effects such as cytokine release syndrome (CRS) and/or immune effector cell-associated neurotoxicity syndrome (ICANS) occur with high incidence and varying degrees of severity, ranging from mild to fatal (Brentjens

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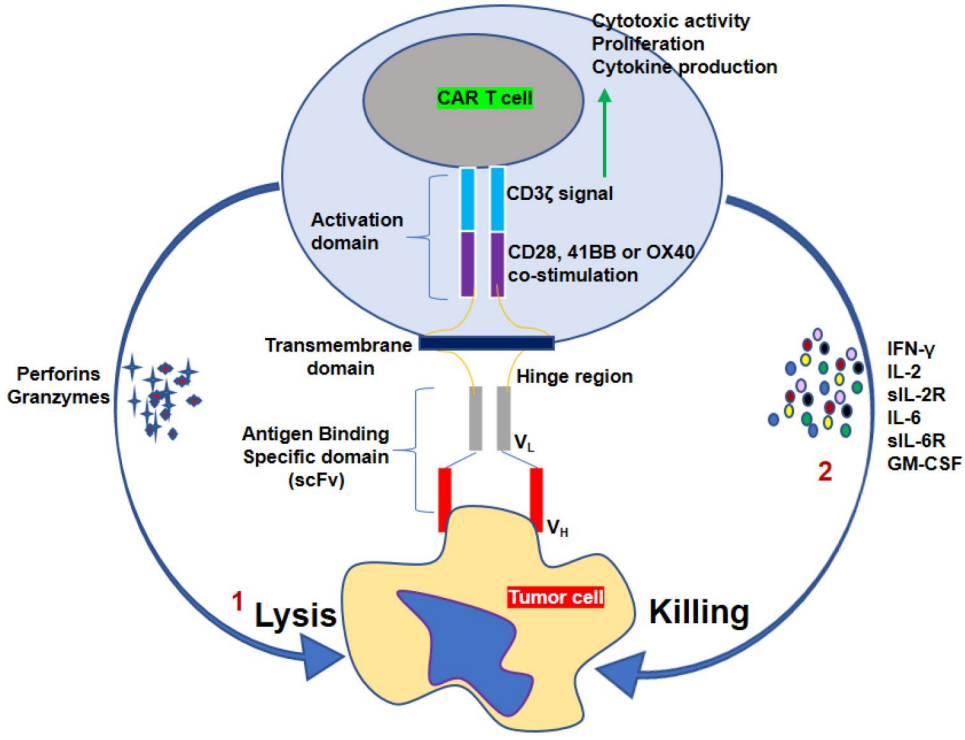

Figure 1. Mode of action of CAR T cells. Binding of the specific antigen on target cells activates antigen-specific CAR T cells. Activated $CD8^+$ CAR T cells produce large amount of granzymes and perforins to exert their cytolytic function, which leads to tumor cell lysis (1). $CD4^+$ as well as $CD8^+$ CAR T cells proliferate and release cytokines (2) upon binding of antigen to the CAR, subsequently leading to the death of tumor cells. CAR, chimeric antigen receptor; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; IL, interleukin; scFv, single-chain fragment variable; sIL-R, soluble interleukin receptor.

et al. 2010; Grupp et al. 2013; Maude et al. 2014; 2018; Shimabukuro-Vornhagen et al. 2018; Curran et al. 2019). The term CRS was first coined in the 1990s after the clinical introduction of the anti-CD3 monoclonal antibody OKT3 (Chatenoud et al. 1989) and reported as a systemic reaction with massive elevations in tumor necrosis factor (TNF)-α and interferon (IFN)-γ. CRS is currently described as a systemic inflammatory response that results from activation/engagement of endogenous or infused T cells and/or other immune effector cells, with progressive symptoms that must include fever at onset, and may include hypotension, hypoxia and organ dysfunction (Lee et al. 2019).

CAR T cell-associated CRS is thought to result from the activation of myeloid cells by highly activated T cells; although anti-interleukin (IL)-6 receptor antibodies (e.g., tocilizumab (Le et al. 2018)) can ameliorate CRS, they do not prevent or treat CRS. A plethora of other cytokines such as IL-1, IL-10, granulocyte-macrophage colony stimulating factor (GM-CSF), and monocyte chemoattractant protein-1 (MCP-1) are involved in CAR T cell-mediated CRS (Wang and Han 2018; Sterner et al. 2019; Morris et al. 2022; Shang et al. 2023) (see Figures 2 and 3). However, it has been difficult to investigate the complex mechanisms underlying the CAR T cell-mediated CRS because most non-clinical models use human tumors in immunodeficient mice that poorly recapitulate the immune system of a patient (Morgan 2012; Rooney and Sauer 2018; Donnadieu et al. 2022). Such a discrepancy between non-clinical testing and clinical observations is a major hurdle for the safe implementation of novel immune therapeutics. More predictive non-clinical test systems are needed to get a better insight in possible safety risks of immunomodulatory drugs.

This review presents the IMI2/EU project imSAVAR (**im**mune **S**afety **A**vat**ar**: nonclinical mimicking of the immune system effects of immunomodulatory therapies) that aims at creating an accessible platform for novel models, tools and resources for the early non-clinical prediction of adverse events in immunomodulatory therapeutics.

irAOP concept exemplified *via* pathophysiology of CRS in CAR T cell therapy as use case

Within imSAVAR, immune-related adverse outcome pathways (irAOP) are defined to guide development of novel test systems for immunomodulatory therapeutics (Tollefsen et al. 2014). AOPs (Adverse Outcome Pathways) are a conceptional framework to describe current knowledge of biological effects triggered by a molecular initiating event (MIE) leading to an adverse outcome (AO) (Ankley et al. 2010). In detail, AOPs describe the link between an MIE and a series of key events (KE), i.e., measurable and essential biological events, at different response levels (e.g., molecular, cellular, tissue, organ, organism) and the respective key event relationship (KER) leading to a specific AO (Ankley et al. 2010; Vinken 2013; Tollefsen et al. 2014; Delrue et al. 2016; Sakuratani et al. 2018).

The AOP framework emerged in the context of regulatory toxicology to guide the effective paradigm shift toward mechanism- and evidence-based chemical risk assessment (Vinken 2013). Rapidly, due to this practical and stressor agnostic approach, the framework gained interest from other fields. AOPs are acknowledged as a useful tool for the design of testing strategies to support safety assessment of nanomaterials and radiation-induced AO (Halappanavar et al. 2020; Chauhan et al. 2021) as well as to systematically organize diverse and fast-evolving knowledge on the COVID-19 pathogenesis (Nymark et al. 2021; Clerbaux et al. 2022).

In the context of immunomodulatory therapeutics, we propose to further expand the scope of the framework and to

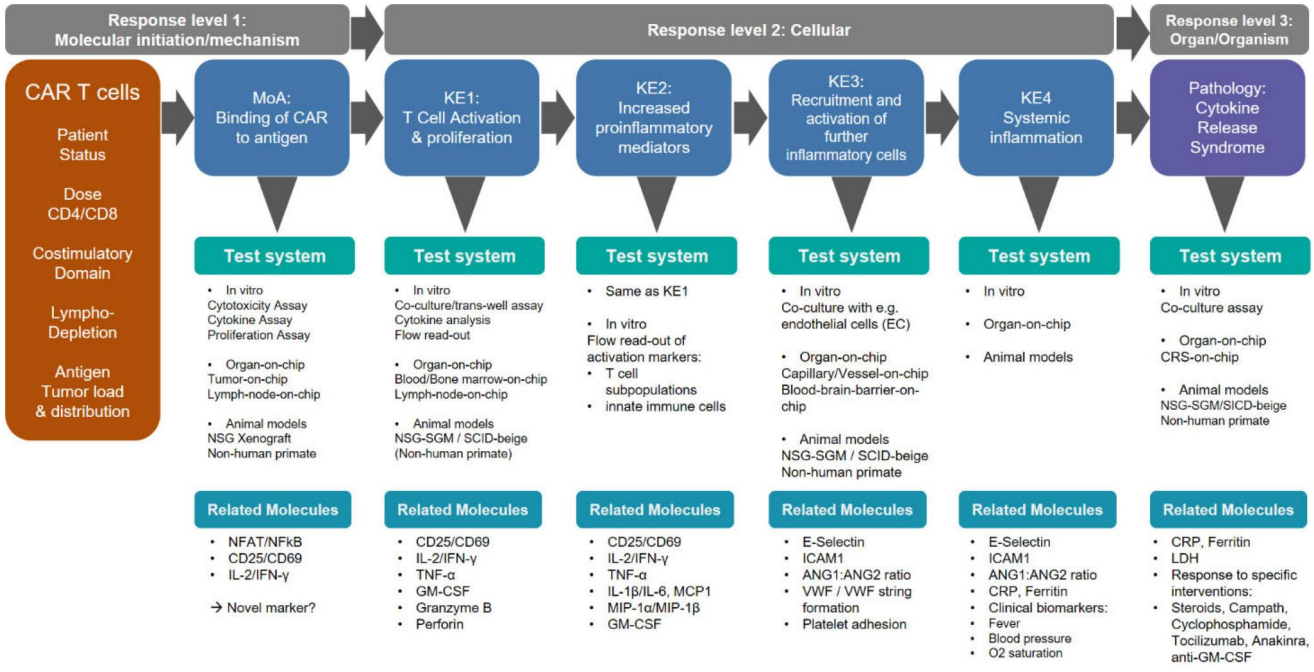

Figure 2. Illustration of the irAOP for CRS mediated by CAR T cells. The binding of CAR T cells to their targets (antigen-expressing cells) represents response level 1 (molecular initiation). The proposed key events (KE) 1-4 represent the cellular response level and may include interactions between different cell types (KE 2 and 3). This cellular response to the initial event (binding of CAR T cells to their target) finally leads to the response on organ and/or organism level (cytokine release syndrome). Possible test systems and a selection of related molecules for the mode of action (MoA; binding to antigen-expressing cells) and the proposed key events are listed. ANG, angiopoietin; CAR, chimeric antigen receptor; CRP, c-reactive protein; EC, endothelial cell; GM-CSF, granulocyte-macrophage colony stimulating factor; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IL, interleukin; KE, key event, LDH, lactate dehydrogenase; MCP1, monocyte chemoattractant protein 1; MIP-1, macrophage inflammatory protein-1; TNF, tumor necrosis factor; VWF, von Willebrand factor.

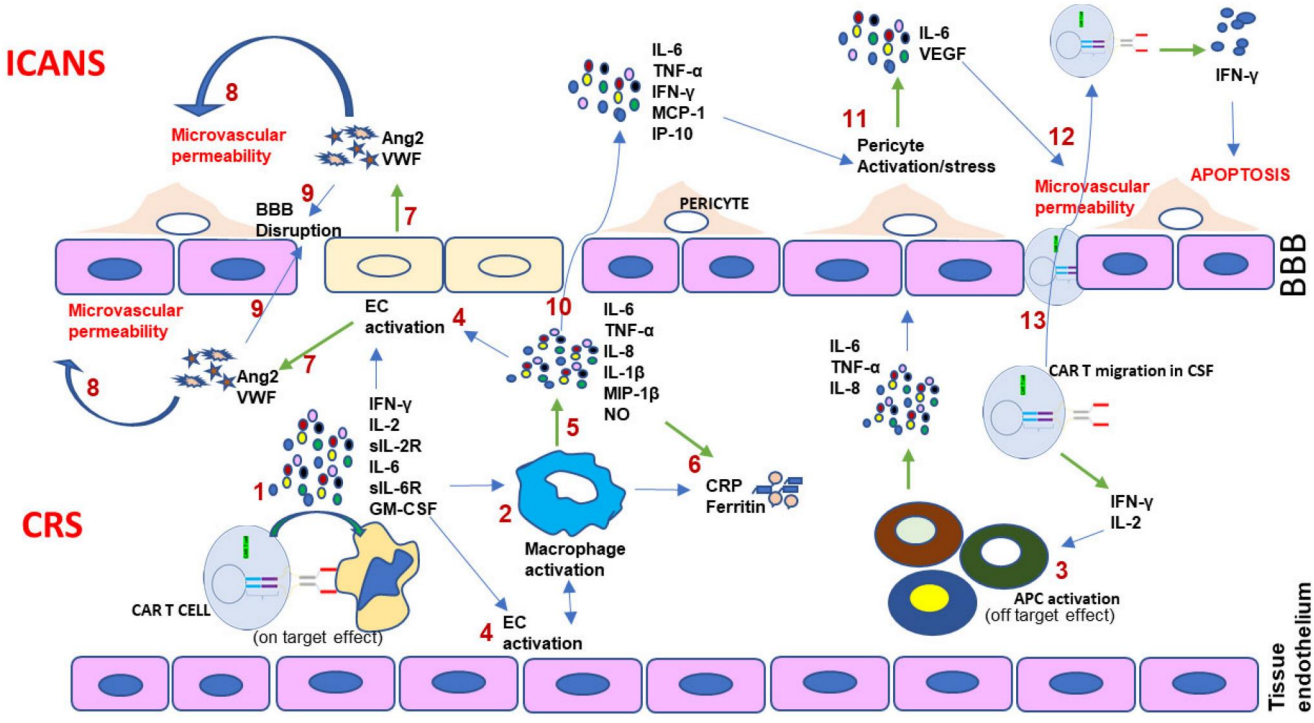

Figure 3. Proposed pathomechanism of CRS and ICANS. Antigen-specific activated CAR T cells (on-target effect) produce inflammatory cytokines (1) resulting in activation of macrophages (2), APC, e.g., dendritic cells, (off-target effect) (3) in the blood circulation and activation of EC (4). Activated macrophages secrete large amounts of pro-inflammatory cytokines (5) contributing to further activation of EC and subsequently produce CRP and ferritin (6). Stimulated EC release VWF and ANG2 (7) associated with CRS and ICANS, resulting in enhanced microvascular permeability (8) and BBB disruption (9). Breach in the BBB allows influx of cytokines into the CNS from blood (10) where these cytokines further activate pericytes (11) which leads to additional IL-6 and VEGF secretion from pericytes. Cytokine storm in the CNS subsequently leads to further enhanced BBB damage and vascular permeability (12) allowing CAR T cell infusion in the brain (13). In the CNS, local cytokines and IFNγ produced by CAR T cell contribute to apoptosis and ultimate neurotoxicity. ANG2, angiopoietin-2; APC, antigen-presenting cells; BBB, blood-brain barrier; CNS, Central nervous system; CRP, c-reactive protein; CRS, cytokine release syndrome; CSF, cerebrospinal fluid; EC, endothelial cell; GM-CSF, granulocyte-macrophage colony stimulating factor; ICANS, immune effector cell-associated neurotoxicity syndrome; IL, interleukin; IFN, interferon; MIP-1, macrophage inflammatory protein-1; NO nitric oxide; sIL-R, soluble interleukin receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; VWF, von Willebrand factor.

exploit irAOPs to link a certain MIE interacting with the immune system and a series of KEs at different biological levels of the immune system leading to an adverse immune response such as excessive inflammation, to CRS as the AO. Structuring KEs along an AOP can provide an instrumental scaffold to anchor relevant test systems. Such an irAOP collects the weight-of-evidence for the MIE, KEs, and KERs, which can also guide the development of test systems to assess the (proposed) KE of a given AO (Selvaraj et al. 2020). Hence, it is the aim to assign suitable *in vitro* and *in vivo* test systems as well as potential/already existing biomarkers for the KE involved. Continuous refinement of such an irAOP will also provide better insight into existing knowledge gaps in given irAOP (Figure 2).

In addition, in contrast to chemical inducing toxicity, the use of an AOP in this context is intended to evaluate the potential of CAR T cell therapies in balancing the efficacy and safety of the treatment. In that context, the quantitative and time dimension along with other biomarkers might be essential to assess the tradeoff between protective and adverse inflammation. Such an irAOP might represent a great opportunity, albeit a challenging one, to clarify within the AOP framework a physiological normal inflammatory response *versus* a pathological excessive inflammation, relevant also to many chemical stressors (Clerbaux et al. 2023).

Table 1 summarizes the Mode of Action (MoA) and KEs of the irAOP for CAR T cell-induced CRS (illustrated in more detail in Figure 3). The binding of the specific antigen on target cells activates antigen-specific CAR T cells enabling them to exert their cytotoxic and cytolytic function by, for example, release of IFNγ and Granzyme B (MoA and KE 1). Furthermore, $CD4^+$ as well as $CD8^+$ CAR T cells proliferate and release cytokines (e.g., IL-2) upon binding of antigen to the CAR (MoA and KE 1). Here, the design of the CAR itself, e.g., the choice of co-stimulatory domain, the transmembrane domain, as well as the nature of the single-chain fragment variable (scFv) binding to the respective antigen, can have a great impact on the function and potency of CAR T cells (Davila et al. 2012; Hudecek et al. 2013; Sadelain et al. 2013; Hudecek et al. 2015; Majzner et al. 2020). The extracellular spacer domain of the CAR can also have an effect on CAR specificity (Guest et al. 2005; Hudecek et al. 2013; 2015; Watanabe et al. 2016). Majzner et al. recently reported that replacement of the CD8 hinge-transmembrane (H/T) region of a 4-1BBζ CAR with a CD28-H/T lowers the threshold for CAR reactivity despite identical signaling molecules (Majzner et al. 2020). This further highlights that different CAR designs also impact on the functionality of CAR T cells in tumors with heterogeneous antigen expression and, hence, also the risk of adverse events like CRS. Further, the intracellular domains of second- and third-generation CARs additionally affect the persistence of CAR T cells *in vivo*, which might also affect the safety of the CAR T cells (Milone et al. 2009; Frigault et al. 2015; Kawalekar et al. 2016; Li et al. 2018). Recently, new strategies in CAR design to avoid tumor escape and toxicities, such as Dual CARs, On-Switch CARs, Universal CARs, or CARs with inducible Caspase-9 (safety switch) were described, all of which can affect the safety of next generation CAR T cell therapy (Diaconu et al. 2017; Guedan et al. 2018). In addition, the cellular composition of the infused CAR T cell product affects both the efficacy and the safety of this immunotherapy (Sommermeyer et al. 2016; Bove et al. 2023).

In addition, as most antigens targeted by CAR T cells are not strictly tumor-specific, e.g., CD19, which is also expressed by many nonmalignant B cells, this may lead to an on-target, off-tumor effect triggering a massive cytokine release by CAR T cells. This in turn may lead to the recruitment of additional (innate) immune cells and trigger the release of cytokines and other pro-inflammatory mediators by these cells (KE 1 and 2). In particular, monocytes and macrophages are described as the main source for several cytokines/chemokines, e.g., IL-1β, IL-6, and MCP-1, which are all attributed to play key roles in the pathophysiology of CRS (Hao et al. 2020; Cosenza et al. 2021). Insights from recently available mouse models also elucidated the role of cytokines (IL-1β, IL-6) and nitric oxide (NO) produced by monocytes and/or macrophages as contributing factors to the development of CRS (KE 3) (Giavridis et al. 2018; Norelli et al. 2018). Recent findings from Liu et al. suggest that granzyme B released from CAR T cells lead to tumor cell pyroptosis, a form of inflammatory cell death, *via* cleavage of Gasdermin E in tumor cells. The pyroptosis of tumor cells trigger IL-6 and IL-1β production in macrophages to induce CRS. Therefore, tumor cell death, in addition to cytokines produced from CAR T cells, could be a factor triggering CRS (Liu et al. 2020). The activation of endothelial cells, which may also produce IL-6 (Obstfeld et al. 2017), contributes to a large extent to the clinical presentation of CRS, namely, capillary leak and hypotension (KE 3 and 4). Clinical parameters of endothelial cell activation include von Willebrand factor (VWF) and angiopoietin-2 (ANG2) which were both recently associated with the severity of CRS in patients treated with CAR T cells (Hay et al. 2017).

The following sections discuss the currently available *in vitro* and *in vivo* test systems, as well as biomarkers for cytokine release and the gaps and limitations of these test systems especially for non-clinical safety assessment of novel CAR T cell products.

In vitro test systems to assess cytokine release by CAR T cells (MIE/MoA, KE1-KE3)

Currently available *in vitro* test systems for cytokine release only partly reflect the complexity of the human immune system and/or the situation at the tumor site, as not all relevant cell types are included or at least not in their physiological ratios. In addition, almost all non-clinical assays "ignore" the influence of lymphodepletion on CAR-T efficacy and safety as well as the influence of the tumor burden (only standardized effector to target ratios) and, importantly, most non-clinical assays are only conducted with cells from healthy donors.

For *in vitro* efficacy testing, CAR T cells are normally co-incubated with antigen-expressing cells or cells that lack antigen expression as a negative control. Here, the cytolytic as well as the proliferative capacity upon antigen encounter can be calculated using standardized effector to target (E:T) ratios which, however, might not reflect the situation *in vivo*. Cytokine release upon antigen encounter has been observed in such co-incubation assays although it is very challenging to draw conclusions regarding the safety of the respective CAR T cell product from these simplistic models.

The addition of for example monocytes or endothelial cells in such co-incubation assays can help to get an insight into the interactions of CAR T cells with target and bystander immune cells and thus provide a potentially-enhanced model to identify potential safety issues (hazard assessment) and investigate associated mechanisms. Ideally, all cell types included in such models would be obtained from the same donor to avoid confounding results due to HLA-mismatch. Such a fully autologous assay was recently described by the Mitchell group. They developed an

Table 1. Mode Of Action of CAR T cells and key events leading to CAR T cell-mediated CRS.

	Description	Strength of Evidence Experimental support	Related molecules
Molecular Initiating Event (MIE)/Mode of Action (MoA)	CAR binding to (tumor) antigen on antigen-expressing cell	Strong: Efficacy of novel CAR T cell products always tested against antigen-expressing cells and cells that do not express target antigen. (Hudecek et al. 2010) Long-lasting remission noted in patients treated with CD19 CAR T cells. (Melenhorst et al. 2022)	IL-2/IFNγ; CD69/CD25; NFAT/NF-κB
KE 1	(CAR) T cell activation and proliferation	Strong for CAR T cells: Cytolytic as well as proliferative capacity upon antigen encounter tested *in vitro* for each CAR T cell product. (Hudecek et al. 2013) Ability to lyse tumors *in vivo* tested in animal models as prerequisite for clinical trials. (Hudecek et al. 2013) Monitoring of CAR T cell expansion during clinical trials. (Hay et al. 2017) Long-lasting remissions in patients treated with CD19 CAR T cells. (Melenhorst et al. 2022)	IL-2/IFNγ; CD69/CD25; Perforin, Granzyme B, TNFα, GM-CSF
KE 2	Increased pro-inflammatory mediators released by (CAR) T cells and bystander (innate) immune cells	Strong for CAR T cells: Ability to release IFNγ and IL-2 upon antigen encounter tested *in vitro* for each CAR T cell product. (Hudecek et al. 2013) Cytokine levels (especially IL-6) in patient sera monitored during clinical application of CAR T cells. (Teachey et al. 2016; Hay et al. 2017) Intermediate-to-Strong for bystander (innate) immune cells: Monitoring of IL-6, CRP and ferritin levels in patient sera. (Teachey et al. 2016; Hay et al. 2017) Role of monocyte- and macrophage-derived IL-1β and IL-6 as mediators of CRS elucidated in recent mouse models of CRS. (Giavridis et al. 2018; Norelli et al. 2018) Recent findings (*in vitro* co-culture experiments, NSG xenograft *in vivo* model) suggest GM-CSF inhibition ameliorates CRS without affecting CAR T cell function. (Sterner et al. 2019; Shang et al. 2023)	IL-2/IFNγ or Multiplex Panel including Perforin, Granzyme B (for CAR T cells); IL-1β, IL-6, NO, IL-8 (for bystander immune cells); clinical parameters like CRP, ferritin, MCP-1
KE 3	Activation of other cell types such as endothelial cells, macrophages, dendritic cells, platelets as well as amplification of cytokine release by CAR T cells, bystander immune cells, endothelial cells	Intermediate-to-Strong: Role of monocyte- and macrophage-derived IL-1β and IL-6 as mediators of CRS elucidated in recent mouse models of CRS. (Giavridis et al. 2018; Norelli et al. 2018) Monitoring ANG2:ANG1 ratio in patients as well as serum levels of IL-6, CRP, and ferritin as well as DIC parameters such as fibrinogen, VWF, prothrombin time, and aPTT. (Teachey et al. 2016; Gust et al. 2017; Hay et al. 2017)	IL-2/IFNγ or Multiplex Panel including Perforin, Granzyme B (for CAR T cells); IL-1β, IL-6, NO, IL-8 (for bystander immune cells); clinical parameters like VWF, ANG2, IL-6, CRP, ferritin, MCP1, DIC parameters like fibrinogen, prothrombin time, aPTT
KE 4	Systemic inflammation	Strong: Increased serum levels of CRP, ferritin and other pro-inflammatory mediators in patients upon CAR T cell infusion. (Teachey et al. 2016)	Multiplex Panel; clinical endpoints like VWF, ANG2, IL-6, CRP, ferritin, MCP1, DIC parameters (see above)
Adverse Outcome	Cytokine Release Syndrome	Strong: CRS is a common adverse event upon CAR T cell infusion. (Brentjens et al. 2010; Grupp et al. 2013; Maude et al. 2014; 2018; Curran et al. 2019)	Same as for KE4

Key Event, KE; ANG, angiopoietin; aPPT, activated partial thromboplastin time; CRP, c-reactive protein; CRS, cytokine release syndrome; DIC, disseminated intravascular coagulation; IL, interleukin; IFN, interferon; MCP-1, monocyte chemoattractant protein-1; NO, nitric oxide; VWF, von Willebrand factor.

autologous endothelial cell/peripheral blood mononuclear cell *in vitro* assay that can detect cytokine responses to, for example, mAb (Reed et al. 2015). In this case, the Authors prepared autologous endothelial cells from peripheral blood progenitors, termed blood outgrowth endothelial cells (BOEC). The BOEC assay is now commercially-available at one partner site of imSAVAR (LabCorp Drug Development Inc.) and is already part of the test concept for the different MoAs covered by imSAVAR, including CAR T cells. This provides a unique opportunity to study things like cytokine release by CD19 CAR T cells and the effect on endothelial cells and other immune cells in a fully autologous *in vitro* test system.

Plans to further improve the BOEC assay to a 3D model are currently under development and will be implemented into the workflow of imSAVAR toward improved non-clinical safety assessment. Recently, the Mitchell group described an advanced version of this BOEC assay ("vessel in a dish" bioassay), where smooth muscle cells were also prepared from progenitor cells present in peripheral blood (Ahmetaj-Shala et al. 2020). Another group recently demonstrated that antigen-presenting cells (APC) secrete IL-6 when co-cultured with CD19-specific CAR T cells and tumor cells expressing CD19. This observation was dependent on the co-incubation of APC with CAR T cells and their respective target cells, but not on cell-cell contact as results could be reproduced in a trans-well assay format, thereby confirming the role of cytokines for the activation/engagement of APC in the CRS pathophysiology (Singh et al. 2017). Another approach was published by Norelli et al. (Norelli et al. 2018) wherein T

cells from newborn hematopoietic stem and progenitor cells (HSPC)-humanized SGM3 mice were used to generate CAR T cells and co-cultured them with either $CD33^{+}CD44v6^{+}$ THP-1 leukemic cells or $CD19^{+}CD44v6^{-}$ BV173 leukemic cells (see also section dealing with 'Animal Models' below).

In addition, whole-blood assays were described in recent years as a low-cost alternative to evaluate cytokine release by mAb like TGN1412 and other immunomodulatory drugs (Langezaal et al. 2002; Damsgaard et al. 2009; Wolf et al. 2012; Finco et al. 2014; Grimaldi et al. 2016). Here, minimally diluted whole blood is co-incubated with the respective drug of interest. Then, after a fixed time period (e.g., 24 hr), plasma is collected and cytokines analyzed. Such an assay is currently not used for studying CAR T cell mediated cytokine release but could be a low-cost method for monitoring cytokines in patient sera. In general, standardization and harmonization of cytokine release assays is still an open issue. The simplest method to analyze cytokine release in supernatants of such co-culture assays would be a solid-phase enzyme-linked immunosorbent assay (ELISA) to analyze for example IFNγ release. For multiplex cytokine analysis, two main formats are available: bead-based liquid-phase assays (such as the BD™ cytometric bead array) and plate-based solid-phase assays (such as the electrochemiluminescence [ECL]-assay from Meso Scale Discovery [MSD]) (Morgan et al. 2004; Dabitao et al. 2011; Castillo and MacCallum 2012).

Assays to measure cytokine release are standardized and validated and are also used in the clinic to measure cytokine levels in human plasma or sera (Badurdeen et al. 2012). In the experimental *in vitro* setting, where - for example - CAR T cells are co-incubated with target cells, such a standardization is missing: E:T ratios may vary, as well as negative and positive controls, culture conditions, incubation times which also vary depending on the MoA used (e.g., CAR T cells vs. bispecific T cell engagers) (Grimaldi et al. 2016). Furthermore, controls are normally included to validate the assay performance and not as a benchmark for a potential safety risk. In addition, it is still difficult to use cytokine levels measured *in vitro* as a guide for the safety of a given immune therapeutic for clinical application (Grimaldi et al. 2016). Therefore, the imSAVAR consortium aims at establishing a "feedback loop" between biomarker development (based on clinical observations) and analyses in 2D/3D *in vitro* models or animal models, which could help to better define cytokine levels that may be harmful. Here, we collect and analyze samples from patients undergoing CAR T cell therapy with FDA/EMA approved CAR T cell products. Here, we will also characterize all immune cell subsets prior and after infusion of the cell product at specific time points.

It should be highlighted that most test systems are still only conducted with cells from healthy donors and permanent (immortalized/tumor) rather than autologous cell lines (primary tumors, patient-derived tumor organoids) (Grimaldi et al. 2016; Hartmann et al. 2016). Still, as patients receiving immunotherapies are normally heavily pretreated, the use of cells from healthy donors may not reflect the cellular distribution of the pathologic condition (Amir et al. 2013). Table 2 summarizes the most widely used assays to analyze cytokine release, examples of controls utilized in these assays as well as their limitations.

Apart from the limitations of such assays in general, additional consideration should be taken into account when using these assays for non-clinical safety assessment of CAR T cell products. Unlike other immune therapeutics such as monoclonal antibodies CAR T cells are a "living drug", i.e., they have the ability to expand and also contract within the body of a patient and can also potentially form a memory pool (Kalos et al. 2011; Park et al. 2018; Melenhorst et al. 2022). This poses a risk of recurrent CRS in case of tumor relapse or on-target/off-tumor toxicity if the targeted antigen is not strictly tumor-specific.

There are also other factors to consider which are (in part) unique for CAR T cells. The antibody fragment (often a scFv of a monoclonal antibody of murine origin) which confers

Table 2. *In vitro* test systems for cytokine release.

Test system	Possible readouts	Controls (examples)	Limitations	Purpose
Co-culture of tumor and immune cells (e.g., CAR T cells)	ELISA Multiplex Cytokine Analysis Flow Cytometry	• Cell culture medium • PMA/ionomycin • Tumor cells lacking antigen expression	Bystander (immune) cells missing	Efficacy testing. Useful for assessment of CAR T cell MoA and KE1/2 (Table 1)
PBMC assay (including high cell density culture "RESTORE" protocol) (Yaqoob et al. 1999; Römer et al. 2011)	ELISA Multiplex cytokine analysis Flow Cytometry	• Cell culture medium • Vehicle controls • PMA/ionomycin • LPS/SEB • Isotype controls • Anti-CD3 (e.g., OKT3) • Anti-CD28 (e.g., TGN1412)	Described for cytokine release of mAb. Might be difficult to translate to non-clinical testing of CAR T cells	Efficacy and safety testing of mAb. Could possibly be adapted for CAR T cells to assess KE2/3 (Table 1)
Co-culture assay of e.g., BOEC or HUVEC and PBMC (Weissmüller et al. 2012; Reed et al. 2015; Gust et al. 2017; Ahmetaj-Shala et al. 2020)	ELISA Multiplex cytokine analysis Flow cytometry	• Cell culture medium • Vehicle controls • PMA/ionomycin • Isotype controls • Anti-CD3 (e.g., OKT3) • Anti-CD28 (e.g., TGN1412)	Confounding results due to HLA-mismatch might occur if cells obtained from more than one donor	Safety testing possible. Could be adapted for CAR T cells to assess MoA, KE1/2/3 (Table 1)
Whole Blood Assay (Langezaal et al. 2002; Damsgaard et al. 2009; Wolf et al. 2012)	ELISA Multiplex cytokine analysis Flow cytometry	• Cell culture medium • Vehicle controls • PMA/ionomycin • LPS/SEB • Isotype controls • Anti-CD3 (e.g., OKT3) • Anti-CD28 (e.g., TGN1412)	Described for cytokine release of mAb. Might be difficult to translate to non-clinical testing of CAR T cells	Safety testing for mAb possible. Might be useful in an adapted version for monitoring of patients during CAR T cell clinical trials.

BOEC, blood outgrowth endothelial cells; CAR, chimeric antigen receptor; ELISA, enzyme-linked immunosorbent assay; HUVEC, human umbilical vein endothelial cells; KE, key event; LPS, lipopolysaccharide; mAb, monoclonal antibody; MoA, mode of action; PBMC, peripheral blood mononuclear cell; PMA, phorbol 12-myristate 13-acetate; SEB, staphylococcal enterotoxin B.

specificity to the CAR, also impacts the affinity of the CAR to its target and therefore on the safety of the CAR (off-target/on-target off-tumor effects or low on/off binding rate, avidity of the CAR) (Majzner et al. 2020). In addition, if not fully humanized, the non-human parts of the scFv might induce an immune response leading to premature elimination of CAR T cells rendering subsequent infusions difficult or even impossible (Turtle et al. 2016; Sommermeyer et al. 2017).

The cellular composition of the final CAR T cell product may also impact the efficacy and hence the safety of CAR T cell therapy. Turtle et al. conducted clinical trials with a defined ratio (1:1) of $CD4^+$ and $CD8^+$ CAR T cells. Here, the Authors found (as also reported by others) that the preconditioning regimen for lymphodepletion also influences persistence, safety and immunogenicity of CAR T cell therapy (Sommermeyer et al. 2016; Turtle et al. 2016; 2016; Maude et al. 2018; Neelapu et al. 2018; Abramson et al. 2019; Schuster et al. 2019; Bove et al. 2023). Further, Globerson Levin et al. reported that by using a mathematically-guided approach, one could test potentially reducing the administered CAR T cell dose in a breast cancer model. The results showed the dose was still efficacious and could be safer as the release of IFNγ was reduced (Globerson Levin et al. 2020).

All these factors presented above may have varying effects on the ability of CAR T cells to induce CRS and have to be taken into account regarding the use of test systems for CRS, i.e., that also controls should be adjusted accordingly. For cytokine release mediated by CAR T cells these controls should be adjusted for CAR design and the scFv targeting the antigen of interest as well as for antigen density on target cells (tumor cells with low or high antigen expression).

Further, most of the above-mentioned *in vitro* test systems mainly focus on measuring released or intracellular cytokines without capturing effects of the interactions of CAR T cells with other blood cells, the endothelium and the target tissue, in other words, the microphysiological environment CAR T cells are experiencing *in vivo*. Here, new advanced *in vitro* test systems like organ-on-chips could be able to close this gap. Such systems are capable of measuring the cytokine release of endothelial cells under varying conditions, and contain a combination of 3D culture, multiple cell types, and flow (Brown et al. 2016; Herland et al. 2016; Biglari et al. 2019).

Advanced *in vitro* test systems to assess cytokine release (MIE/MoA, KE1-KE4)

Organs-on-chips (OoC) are defined as "fit-for-purpose" microfluidic devices, containing living engineered organ substructures in a controlled microenvironment, that recapitulates one or more aspects of the organ's dynamics, functionality and (patho)physiological response *in vivo* under "real-time monitoring" (Mastrangeli et al. 2019b). Using advanced bioengineering approaches, OoC mimic physiologically-relevant tissue microenvironments and recapitulate the interaction between different human cell types and the interaction of different tissues. Most importantly, a completely controlled microenvironment that requires very few cell numbers allows for testing hypotheses in the presence or absence of specific cell types and for comparing patient-specific reactions relative to the physiological response of tissues from healthy individuals (Mastrangeli et al. 2019a).

OoC development and use has grown exponentially over the past decade (Mastrangeli et al. 2019b) and, in the field of small molecule toxicity, they have been shown to recapitulate key *in vivo* human features that were neither recapitulated in classical *in vitro* systems, such as multi-organ toxicokinetic studies (Gholizadeh et al. 2022; Shroff et al. 2022; Maass et al. 2019; McAleer et al. 2019) or immune cell recruitment (Kerns et al. 2021; Cipriano et al. 2022; Rogal et al. 2022) nor in animal models (Jang et al. 2019; Richardson et al. 2020; Koning et al. 2021; Bjornson-Hooper et al. 2022; Ingber 2022). The most common OoC systems tested for drug toxicity applications represent tissues such as liver, gut, lung and heart. More recently, several cancer-on-chip models have been developed (Pavesi et al. 2016; Sleeboom et al. 2018; Sontheimer-Phelps et al. 2019) but the use of cancer-on-chip models combined with immune cells is still scarce (Lee et al. 2018; Maulana et al. 2021). However, for the study efficacy and safety of immunotherapies, models that include relevant features of the immune system as well as the vasculature are essential (Maulana et al. 2021; Cipriano et al. 2022). The endothelium is the first contact point for the circulating immune cells and their released cytokines. It, therefore, plays a key role in the mechanisms of toxicity of immunotherapies. Many OoC studies already employ methods that can cover the proposed irAOP for CAR T cells from KE1 to KE4. The systemic inflammatory response to CAR T cell therapies (KE4) is influenced by the changes that a treatment might cause on the activation of the endothelium and the resulting cytokine release (KE2 + 3). Moreover, the adverse outcome on an organismal level (hypotension, capillary leak, microthrombi) is caused for an important part by endothelial dysfunction (e.g., loss of barrier function) (KE3). A number of OoC have been introduced focusing on recapitulating functional vascular endothelium (Huh et al. 2010; Griep et al. 2013; Abaci et al. 2014; Herland et al. 2016).

Endpoints such as the capacity to retain the controlled permeability to large molecules, the release of cytokines, the presence of endothelial cell adhesion markers, and the adhesion of platelets and monocytes are commonly analyzed while stimulating with stressors that are known to activate the endothelium and/or to disrupt the membrane (Brown et al. 2016; Herland et al. 2016; Biglari et al. 2019). Co-perfusion of tumor cells and monocytes through an OoC model integrating a self-organized 3-D vascularized network of endothelial cells has enabled the investigation of the interplay between tumor cell and monocytes during extravasation (Boussommier-Calleja et al. 2019). The co-perfusion with immune cells is also a key feature necessary to study the safety profile of CAR T cells and to bridge KE1 with KE2 and for studying the KE2/3 interplay. A different study co-cultured immune cells in a tumor-on-chip model in a static, not perfused configuration and tested the effect of trastuzumab on the immunosuppressive mechanisms of cancer associated fibroblasts (CAF) using time-lapse imaging, advanced video analysis and 3-D live images (Nguyen et al. 2018). Those techniques allow for a qualitative and quantitative assessment of the interaction of the immune cells with the cancer cells and could be applied to study KE3/KE4. The microfluidic character of OoC ensures a more physiological cell-to-media-ratio essential to assure relevant concentrations of cytokines as well as an effective and physiologically relevant paracrine signaling between different cells within the same tissue. Overall, tailored OoC systems that allow the evaluation of barrier function, cell adhesion to endothelium, cell migration and invasion as well as a relevant production of cytokines will allow the study of CAR T cell irAOP (Table 3). This can be done either in a stepwise approach, one device tailored for each KE, or in a holistic model representing the KE1/2/3/4. The controlled nature of OoC models would also enable studies elucidating the role of each individual cell type for a specific KE and the final adverse outcome.

Table 3. OoC Systems for CAR T cell efficacy and safety assessment.

Test system	Possible readouts	Controls (examples)	Limitations	Purpose
Cancer-chip	Imaging (monitor cell migration) Multiplex Cytokine Analysis	• Cell culture medium, conditioned • Tumor cells lacking antigen expression • Healthy cells vs cancer cells (positive control)	Bystander (immune) cells missing	Efficacy testing. Useful for assessment of CAR T cell MoA and KE1/2 (Table 1)
Vascular-chip	Live imaging (monitor membrane integrity) Multiplex Cytokine Analysis Platelet/monocyte adhesion	• Cell culture medium, conditioned from cancer-chips • Membrane disruptors • Endothelial cells stressors (e.g., TNF-α, as positive control)	Cancer cells are missing	Safety testing possible. Could be adapted for CAR T cells to assess MoA, in a patient specific fashion KE2/3 (Table 1)
Vascularized cancer chip	Live imaging (monitor membrane integrity) Imaging (monitor cell migration) Multiplex Cytokine Analysis	• Same as above	To evaluate the order of the observed events	Safety testing possible. Could be adapted for CAR T cells to assess MoA, in a patient specific fashion KE1/2/3 (Table 1)
Vascularized cancer chip connected to the vascular chip	Live imaging (monitor membrane integrity Imaging (monitor cell migration) Multiplex Cytokine Analysis	• Same as above • Combinations where the exposure occurs first on the vasculature and/or first on the cancer tissue		Safety testing possible. Could be adapted for CAR T cells to assess MoA, in a patient specific fashion KE1/2/3/4 simultaneously (Table 1)

CAR, chimeric antigen receptor; KE, key event; MoA, mode of action.

A detailed mechanistic approach using tailored OoC models, at the molecular level, will also support the discovery and validation of biomarkers in early stages leveraging a human-relevant but controlled environment. However, very complex OoC models reflecting interactions between various immune cells and the respective cancer cells are still under development (Maulana et al. 2021). The main limitation of applying OoC models for CAR T cell efficacy and safety assessment is the possibility of missing off-target effects in organs not included in the study design. Particularly, KE4, systemic inflammation and the KE relationships thereof, could be incomplete. Here, animal models could allow a more explorative approach in the sense that all organs can be analyzed; however, only if all relevant human targets are indeed expressed (Bjornson-Hooper et al. 2022).

Animal models (non-clinical assessment of CRS mediated by CAR T cells *in vivo*)

In recent years, several syngeneic mouse models for CD19 CAR T cell therapy have been described. In the model reported by Kochenderfer et al. murine CD19 CAR T cells were able to eradicate $CD19^+$ lymphoma as well as normal B cells (B cell aplasia). However, no side effects due to CAR T cell therapy were reported in this model (Kochenderfer et al. 2010). Davila and colleagues described a similar model using C57BL/6 mice. Here, murine CD19 CAR T cell therapy induced long-term remission and B cell aplasia in mice engrafted with acute lymphoblastic leukemia (B-ALL). However, similar to the Kochenderfer mouse model, no signs of CRS like weight loss or elevated cytokine levels were reported in CD19 CAR T cell treated mice (Davila et al. 2013). Pennell et al. reported recently a human CD19 transgenic mouse model where CD19 CAR T cells not only induced B cell aplasia but also toxicities associated with CAR T cell therapy. Mice treated with CD19 CAR T cells showed signs of CRS like weight loss which could be blunted with IL-6 neutralizing therapy (Pennell et al. 2018). Hence, this model could be a useful tool for studying CD19 CAR T cell-associated toxicities. However, as the murine immune system differs from the human system, e.g., in CD28 downstream signaling (Porciello et al. 2018), translation of experimental CAR T cell therapy in syngeneic/transgenic mouse models to the situation in patients might be difficult to achieve. Translation to the bedside could also be hampered if CAR vectors used to treat mice differ from CAR vectors used in human *in vitro* studies and/or clinical trials.

Use of established cell lines for tumor engraftment might also be a limiting factor as fresh tumor samples are more heterogeneous as cell lines passaged *in vitro* for a long time. Hence, patient-derived tumor xenograft models (PDX models) could be a useful tool for non-clinical CRS assessment (Hidalgo et al. 2014; Byrne et al. 2017). Here, use of patient-specific organoids in advanced 3D models could also be a way to circumvent the use of established cell lines in tumor models (Maulana et al. 2021). Norelli et al. were able to mimic CRS in humanized triple transgenic NSG mice (HuSGM3) engrafted with a patient-derived $CD19^+$ ALL-CM leukemia cell line. This model also elucidated the role of monocyte-derived IL-1β and IL-6 as mediators of CRS, which could be prevented by monocyte depletion (Norelli et al. 2018).

Recently, Giavridis and colleagues reported an improved SCID-beige mouse model mimicking CRS upon CD19 CAR T cell therapy (Giavridis et al. 2018). As the incidence of CRS is also associated with tumor burden (Davila et al. 2014; Maude et al. 2014; Turtle et al. 2016; Park et al. 2018), these Authors engrafted mice intraperitoneally with Raji lymphoma cells. Cells were allowed to grow to vascularized solid tumor masses before injection of CD19-specific CAR T cells. Here, CRS developed within three days after CAR T cell treatment and was mediated by macrophages producing IL-1β, IL-6 and NO (Giavridis et al. 2018).

Pfeiffer et al. developed an NSG mouse model with *in vivo* generation of CD19 CAR T cells by using a CAR vector specifically targeting $CD8^+$ T cells (Pfeiffer et al. 2018). Here, CAR expression in $CD8^+$ T cells resulted in B cell depletion and in some mice signs of CRS could be detected. As this model relies on the use of a special CAR vector the translatability to other CAR targets might, however, be limited. Van der Stegen and colleagues used a SCID-beige mouse model to evaluate the toxicity associated with a target that is also expressed in healthy tissues

(off-tumor toxicity). They developed an ErbB CAR that could target human and mouse ErbB dimers also expressed in healthy tissues. Here, toxicity of CAR T cell therapy was associated with the release of IL-6 by macrophages. This was also depending on the site of CAR T cell administration (van der Stegen et al. 2013). Such a model might be a useful tool if the specific CAR is able to target both - the intended human target cells as well as healthy murine tissues expressing the target at physiological levels.

Taken together, currently available mouse models can only reflect the situation in patients suffering from CRS upon CAR T cell administration in a limited fashion. One major limitation is the use of immunocompromised mouse models like non-obese diabetic/severe combined immunodeficiency (NOD-SCID)-γc^{-} (NSG) mice (Ito et al. 2002; 2012). Such mouse models are a useful tool for efficacy testing of novel cell products as they tolerate the engraftment of tumor cells and other cell types like HSPCs of human origin (Ishikawa et al. 2005; Shultz et al. 2005; Brehm et al. 2019). However, without engraftment of HSPCs or cells of similar origin mimicking at least in part the human immune system, such models do not allow studying possible immune-related adverse events of the administered therapy. Syngeneic models as exemplified above using immunocompetent mice can overcome this obstacle. Here, however, the CAR T cells need to be of murine origin, thereby possibly limiting the translatability to the human setting.

Use of dogs as a large animal model for CAR T cell therapy was also recently discussed as dogs were originally used to study efficacy of hematopoietic stem cell transplantation. The high incidence of spontaneous tumor development in outbred dogs could here be of advantage in testing novel CAR T cell products (Mata et al. 2014; Panjwani et al. 2016; Migliorini et al. 2018). As there however might be also (unknown) differences between canine and human immune systems, translatability of such studies might be limited.

The similarity between the human and non-human primate (NHP) immune system potentially allows for safety testing of novel CAR T cell products. However, human CAR T cells engrafted into NHP host are not only at the risk of graft rejection by the host, but also lead to graft versus host disease. To avoid such risk, autologous CAR T manufactured with NHP cells have been developed (Berger et al. 2015; He et al. 2017; Siegler and Wang 2018; Taraseviciute et al. 2018). However, use of NHP CAR T cells is not relevant in predicting CRS risk by human CAR T cells. In addition, there are significant difficulties in generating functioning NHP CAR T cells by lentivirus, due to species-specific restriction factors such as rhesus TRIM5α in NHP (Berger et al. 2015; He et al. 2017; Siegler and Wang 2018; Taraseviciute et al. 2018). Moreover, NHP models are immunocompetent, and do not allow for engraftment of tumors. Therefore, the value of NHP models in predicting CRS risk by CAR-T therapies is limited, and NHP studies have not been leveraged as an integral part of CAR-T investigational new drug (IND)-enabling safety assessment packages (Lebrec et al. 2021). Table 4 summarizes currently available animal models for CAR T cells and their limitations regarding translation and/or CRS assessment.

As outlined in the section dealing with '*in vitro* test systems' above, in all these models presented here, appropriate controls should also be taken into account for CAR design (persistence, production of cytokines), use of scFv (affinity, antigen density on target cells, possible off-target toxicity) and the composition of the infused CAR T cell product.

Table 4. Currently available animal models for CAR T cell therapy.

Model	Description	Limitations	Purpose
Syngeneic mouse model (Kochenderfer et al. 2010; Davila et al. 2013)	Immunocompetent model. Tumor cells and CAR T cells of murine origin.	Translation to situation in patients might be limited.	Efficacy and safety testing of CAR T cells (off-tumor toxicity). Assessment of MoA and KE1/2 possible (Table 1).
NSG mouse model (Ito et al. 2002)	Immunocompromised model lacking T, B, NK cells. Tumor cells are engrafted followed by CAR T cell administration (both of human origin).	Without immune cell engraftment, CRS assessment not possible.	Efficacy testing of CAR T cells (MoA, KE1). Safety testing possible if mice are also engrafted with human immune cells (MoA, KE1/2).
SCID-beige mouse model (Giavridis et al. 2018)	Engraft 3 x 10^6 Raji tumor cells (IP injection of lymphoma cell lineof human origin). After 3 wk, IP injection of 30 x 10^6 CAR T cells (human origin).	Artificial tumor site. Engraftment of tumor in bone marrow ('physiological' tumor niche) might give different results.	Efficacy and safety testing of CAR T cells (CRS). Assessment of MoA, KE1-4 possible (Table 1).
HuSGM3 mouse model (PDX model) (Norelli et al. 2018)	Sublethally irradiated newborn triple transgenic NSG mice engrafted with HSPC and ALL cells of human origin. Four or seven weeks (low or high tumor burden) later, administration of CAR T cells of human origin.	Availability of matched HSPC and tumor cells might be limited.	Efficacy and safety testing of CAR T cells (CRS). Assessment of MoA, KE1-4 possible (Table 1).
Dogs (Mata et al. 2014; Panjwani et al. 2016; Migliorini et al. 2018)	Immunocompetent model. Tumor cells and CAR T cells of canine origin.	Ethics, costs, only specialized centers, smaller groups due to animal size. With dogs possibly limited translatability to humans.	Efficacy and safety testing of CAR T cells (off-tumor toxicity). Assessment of MoA and KE1/2 possible (Table 1).
Non-human primates (Berger et al. 2015; Siegler and Wang 2018)	Immunocompetent model. CAR T cells of primate origin.	Ethics, costs, only specialized centers, smaller groups due to animal size. GVHD and graft rejection, if using human CAR T cells. Poor lentivirus CAR transduction of NHP cells and lack of relevance if using NHP CAR T cells. No tumor.	Efficacy and safety testing of CAR T cells (off-tumor toxicity). Assessment of MoA and KE1/2 possible (Table 1).

ALL, acute lymphoblastic leukemia; CAR, chimeric antigen receptor; GVHD, graft-vs-host disease, HSPC, hematopoietic stem and progenitor cell; IP, intraperitoneal; KE, key event; MoA, mode of action, NHP, non-human primate.

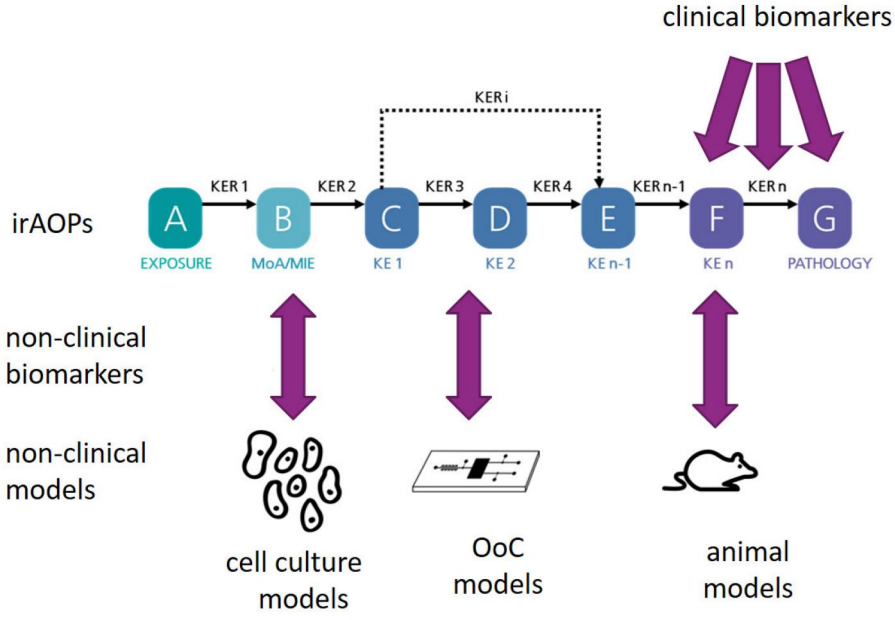

Figure 4. Schematic overview. The overview depicts biomarkers and connectors between irAOPs and non-clinical safety models to support the iterative development of non-clinical safety models. Biomarkers are biological characteristics describing the underlying immunological processes leading eventually to toxicities and serve as indicators for the suitability of a non-clinical safety model. irAOP, immune-related adverse outcome pathway; KE, key event; KER, key event relationship; MIE, molecular initiating event; MoA, mode of action; OoC, organ-on-chip.

Biomarkers for toxicities in CAR T cell therapy

Following the Biomarkers Definition Working Group (Biomarkers Definitions Working Group 2001) (Biomarkers Definitions Working Group 2001), biomarkers for non-clinical safety assessment of immunotherapeutic interventions like CAR T cells are defined as *measurable and evaluable biological characteristics* evaluating the MIE, the KE or the adverse event (e.g., CRS or ICANS) of an irAOP. In addition, biomarkers support the evaluation of non-clinical models assessing safety of novel CAR T cell constructs. Non-clinical models for safety assessment of novel CAR T cell constructs should mimic as closely as possible immunological processes underlying the MIE, KE and/or adverse events of CAR T cells. In order to be able to evaluate this, non-clinical models must objectively measure related biomarkers in a repeatable and reproducible manner. Biomarkers for non-clinical development of CAR T cells are either indicators for the underlying immunological processes leading eventually to toxicities or for the suitability of a non-clinical safety model. However, biomarkers may also be correlated with the occurrence of CRS without being involved directly in the CRS pathophysiology but can be used to predict if there will be an increased risk of developing CRS. Thus, biomarkers support iterative improvement of AOPs and non-clinical safety models (Figure 4).

The majority of reported biomarkers related to CRS and ICANS triggered by CAR T cells are clinical biomarkers, i.e., biological characteristics used for early prediction of adverse events in patients and used for patient management (Teachey et al. 2016; Hay et al. 2017; Wang and Han 2018; Dholaria et al. 2019). Teachey et al. assessed 39 children and 12 adults with refractory/relapsed ALL treated with CTL019 using a preselected panel of 43 cytokines and compared cytokine levels between mild and severe grades of CRS. Among those, 24 cytokines (e.g., IFNγ, IL-6, IL-8, sIL-2Rα, sgp130, sIL-6R, MCP1, MIP1α, MIP1β, and GM-CSF) depicted statistically significant changes and combined in a selection of different statistical models (e.g., logistic regression and decision tree) to predict severe CRS. However, even though the serum biomarkers CRP and ferritin were related to CRS, they did not add informative value to models predicting severe CRS (Teachey et al. 2016). Hay et al. assessed 133 adult patients receiving CD19 CAR T cells to predict CRS using a preselected set of potential biomarkers (Hay et al. 2017). Among several different clinical parameters, cyclophosphamide/fludarabine lymphodepletion, cytokine levels, serum angiopoietin-1 (ANG1), ANG2, and VWF levels were measured in a subset of patients. Higher levels of VWF and ANG2:ANG1 ratio were significantly associated with CRS Grade ≥ 4 compared to Grades < 4. However, biological characteristics for early prediction of CRS Grade ≥ 4 compared to Grades < 4 comprise cytokines IFNγ, IL-6, IL-8, IL-10, IL-15, MCP-1, TNF receptor p55 (TNFRp55), and macrophage inflammatory protein-1β (MIP-1β). A classification tree model for early identification of high-risk patients included fever and levels of MCP1 as parameters. The same model with the additional parameter IL-6 is predictive for high grade neurotoxicity (Gust et al. 2017). Rossi et al. used a panel of 32 secreted proteins to assess up to 2000 single cells of the pre-infusion product of CD19 CAR T cells and defined five functional profiles of CAR T cells (Rossi et al. 2018). Interestingly, polyfunctional CAR T cells (i.e., CAR T cell products with at least two proteins from different functional profiles to be co-secreted in single cells) were associated with Grade ≥ 3 CRS.

Even though a plethora of molecules is known to be clinically related to toxicities triggered by CAR T cells (see above) and can partly be assigned to KEs of the related irAOP (see Table 1 and Figure 2), no standards are available to assess their suitability for application in non-clinical development of novel CAR T cell constructs. Hence, transferring reported clinical biomarkers to non-clinical assessment of novel CAR T cells requires addressing the following questions among others:

i. Which molecules are objectively measuring and evaluating the immunological processes underlying KEs leading

eventually to CRS or ICANS, i.e., are reproducible indicators for these biological processes?;

ii. Which of those described molecules are objectively measurable and evaluable as readouts of non-clinical models for CRS or ICANS triggered by CAR T cells?;

iii. Which observed changes of molecule abundancy of an upstream KE are critical leading to measurable and evaluable changes in the downstream KE, and thus eventually triggering harmful CRS or ICANS in first-in-human studies?; and,

iv. Which molecules are robust and generalizable indicators for immunological processes independent from technical variations among different instances of the same non-clinical model?

Changes in (serum) cytokine levels are especially important biological characteristics as indicators for the risk of harmful CRS (Teachey et al. 2016; Dholaria et al. 2019). However, the inter-individual variability of baseline cytokine levels as well as their association to diverse pathogenic processes make it difficult to identify a specific set of cytokines for separate KEs and to define a threshold for toxicity (Tarrant 2010), i.e., leading to measurable changes in downstream KEs. The majority of molecules herein described to be related to toxicities of CAR T cells (see Table 1 and Figure 2) were detected based on studies assessing a preselected list of marker candidates. However, utilizing unbiased approaches for future biomarker discovery has the potential to reveal still unknown immunological characteristics leading to severe CRS or ICANS.

To explore CRS in an unbiased manner Norelli et al. (Norelli et al. 2018) performed single-cell RNA sequencing (scRNA-seq) of whole human $CD45^+$ leukocytes isolated from leukemic humanized mice treated with CAR T cells. Twelve major lymphoid and myeloid cell populations were identified based on cluster analysis at Day 2 and Day 7 after fever onset. They showed that on single-cell level, monocytes are the major source of inflammatory cytokines, including IL-1β and IL-6 as well as CXCL8 (IL-8), CCL2 (MCP1), CCL8 and CXCL10. Besides monocytes, other cell types, including dendritic cells, also contributed to inflammatory cytokine production. Sheih et al. used single cell transcriptomics and TCRB sequencing to analyze the clonal diversity of CAR T cells at various times following infusion in patients with acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma and different grades of neurotoxicity and CRS. Unsupervised clustering of the scRNA-seq data identified two out of four clusters from clones at early time points after infusion that were enriched with genes associated to cytotoxicity and proliferation states, while at later time points after infusion proliferation states declined without enrichment of an exhaustion gene signature. However, they did not relate their findings to CRS or ICANS (Sheih et al. 2020).

Li et al. performed time series transcriptome profiling in one plasma cell leukemia patient (grade 3 CRS) treated with B-cell maturation antigen (BCMA) CAR T cells. Those authors analyzed scRNA-seq datasets including infusion products, CAR T cells, and endogenous T cells at the peak and remission phases. Gene signatures of the infusion products were enriched in metabolically active, glycolysis and biosynthetic pathways. At the peak phase, two intermediate states were observed: a highly proliferative and a cytotoxic state, assuming a transition from proliferation to cytotoxic activity, whereas CAR T cells in remission phases were in a "resting primed" memory-like state (Li et al. 2021). Moreover, the Authors re-analyzed the single-cell datasets from Sheih et al. and found that BCMA- and CD19-directed CAR T cells had similar transcriptional characteristics, especially at the CAR T cell peak phase. Deng et al. performed whole transcriptome scRNA-Seq of infusion products for 24 patients with large B-cell lymphoma (Deng et al. 2020): in patients who were less responsive to treatment, an enrichment for exhausted T cells was observed, whereas those subjects who achieved complete response showed enrichment for memory T cells. For patients with high-grade CRS, the Authors observed a negative association with exhausted $CD8^+$ T cells and a positive association with exhausted $CD4^+$ T cells. Moreover, based on scRNA-seq and hybrid capture sequencing, they found cell populations with transcriptional expression pattern similar to myeloid lineage, which was associated with development of high-grade neurotoxicity. These ICANS-associated cells expressed genes including *IL1β* and *CXCL8* (*IL-8*), which were related to CRS (Norelli et al. 2018).

By performing a transcriptome analysis using human brain scRNA-Seq and bulk RNA-Seq datasets as well as immunohistochemistry, Parker et al. showed that CD19, regarded as B cell specific, was expressed on mural cells, which line blood vessels and maintain the blood-brain barrier (BBB) integrity (Parker et al. 2020). Based on mouse CD19-directed CAR T cells they indicated that neurotoxicity may be mediated by targeting $CD19^+$ mural cells in mice due to increasing the BBB permeability. However, the study did not determine the precise contributions of CRS in this process. Fraietta et al. revealed by transcriptomic profiling, that CD19 CAR T cell products from complete responding patients with chronic lymphocytic leukemia were enriched in memory phenotype, including IL-6/STAT3 signatures, whereas those who did not respond showed enrichment for pathways including effector T cell differentiation, glycolysis, exhaustion and apoptosis (Fraietta et al. 2018). Performing RNA-seq and miRNA-seq analysis of bone marrow cells from four patients with B-ALL before and after CD19 CAR T cell therapy, resulted in a global view of the transcriptome profiles and regulatory mechanisms in activated CAR T cells (Zhang et al. 2019). In one out of the four patients with severe CRS (the others achieved a molecular remission) up-regulated differentially expressed genes (DEG) were enriched in cytokine-related and acute inflammatory response processes, while the down-regulated DEG were enriched in the apoptosis and endocytosis related pathways.

Comprehensive single-cell or bulk transcriptome analyses using CAR T cells also provide an opportunity to guide the evaluation and optimization of CAR T cell manufacturing or correlate CAR T cell specific molecular signatures with clinical outcomes. To elucidate the underlying mechanisms of cytokine profiles, Xhangolli et al. used three different assays (Xhangolli et al. 2019). Co-cultured CAR T cells from healthy donors with Raji lymphoma cells were analyzed by single-cell 3′ mRNA transcriptome sequencing, multiplexed single-cell cytokine secretion assay and live cell imaging of cytolytic activity. The Authors illustrated the polyfunctional response of CAR T cells, whose activation states are highly mixed with T-helper cell (T_H) Type 1, T_H2, and regulatory T (T_{reg}) cell functions. Cytotoxic activities were equally observed in both $CD4^+$ and $CD8^+$ CAR T cells associated with a wide range of T_H1 and T_H2 signature cytokines (e.g., IFNγ, TNFα, IL-5, IL-13). They showed that GM-CSF is highly prevalent in activated $CD4^+$ CAR T cells independent of differentiation. Boroughs et al. showed that 4-1BB-based CAR T cells were enriched in a central memory phenotype compared with CD28-based CAR T cells which could have profound effects

on the heterogeneity and functional state of CAR T cell infusion products (Boroughs et al. 2020). Wang et al. performed single-cell profiling of APRIL CAR T cell products of three healthy donors in the presence and absence of antigen-specific stimulation and the resulting molecular signature for CAR T cell activation provides a resource for future dissection of underlying mechanisms (Wang et al. 2021).

Conclusions and outlook

The success of CAR T cell therapy in the treatment of relapse/refractory B cell malignancies has paved the way for the development of novel CAR T cell products targeting also e.g., multiple myeloma and solid tumors (Scheller et al. 2024). The high remission rates, however, come with the price of potentially fatal side effects like CRS. Currently available non-clinical *in vitro* as well as *in vivo* models often fail to reflect the complexity of the human immune system and are therefore not able to accurately predict possible harmful side effects like CRS. Furthermore, standardized biomarkers to measure or even predict the occurrence of CRS are still missing. In addition, simplistic models with limited read-outs and endpoint analysis might underestimate potential adverse effects of novel immune therapeutics.

The IMI2/EU project imSAVAR develops immune-related adverse outcome pathways (irAOPs) which shall guide the refinement and/or development of non-clinical models to assess the safety of novel immunomodulatory therapies (Mazein et al. 2023). With the help of the irAOP concept we aim at establishing a platform with refined test systems and biomarkers to predict possible serious side effects of novel immunotherapies. Further, we aim at establishing a model grading system, which may be used as a guide for researchers who want to include safety assessment in their non-clinical analysis. In addition, the effects of established/novel interventional therapies could also be assessed on such a test system platform.

Furthermore, more sophisticated 3D *in vitro* models like organs-on-chips could in the future bridge the gap between safety assessment in "classical/conventional" *in vitro* test systems and in animal models thereby also reducing the costs for the development of novel immune therapeutics. This could in future also lead to a replacement of animal models by complex OoC models with advanced non-clinical biomarkers based on the irAOP. In complex OoC models the influence of additional cell types, e.g., fibroblasts in solid tumors, on the development of CRS and other immune-related adverse events could also be studied in more detail (Maulana et al. 2021).

Current clinical management of adverse outcomes of CAR T cell treatments builds on a minor number of statistical models interrogating biomarkers and general indications (like fever), but are mostly trained with a non-representative sample size and lack validation in independent patient cohorts. Due to limited mechanistic understanding of the processes triggering adverse outcomes of novel engineered T cell constructs, risk prediction of adverse outcomes early in development of these constructs is still hard to achieve. The framework of each irAOP supports sharing and continuously updating a common understanding of the molecular and cellular processes leading to adverse outcomes, and thus supports systematic biomarker development. In this journal issue, Mazein et al. take this approach even further by implementing the linear irAOP into an interactive platform thereby enabling an extended exploration of the underlying molecular mechanisms. Further, with increasing availability of advanced methods for examining molecular and cellular states (e.g., multi-omics or multi-parametric flow cytometry, single-cell where needed) as well as with new tools for integrative bioinformatics changes in the underlying processes of KEs and KERs can be easier described in the future. Therefore, we propose, to integrate advanced (unbiased) assessment of molecular and cellular states into non-clinical development of novel CAR T cell constructs. This will pave the way to an iterative approach to improve understanding of pathogenesis and occurrence of adverse outcomes, to improve non-clinical safety models and to define novel biomarkers predicting risk of adverse outcomes in first-in-human studies.

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Declaration of interest

The authors declare no competing interests.

Disclamier

This publication only reflects the personal views of the stated authors. Neither IMI nor the European Union, EFPIA, or JDRF INTERNATIONAL are responsible for any use that may be made of the information contained therein.

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